

IV.E.3 Photobiological H₂ Production Systems: Creation of Designer Alga for Efficient and Robust Production of H₂ from Water

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University of Missouri, Columbia, Missouri

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Projected End Date: September 30, 2015

Objectives

- Develop advanced renewable photobiological hydrogen production technologies through creation of a designer alga by genetic insertion of a proton channel into an algal thylakoid membrane.
- By 2015, demonstrate an engineering-scale biological system that produces hydrogen at a plant-gate cost of \$10/kg projected to commercial scale.

Technical Barriers

This project addresses the following technical barriers from the Hydrogen Production section of the Hydrogen, Fuel Cells and Infrastructure Technologies Program Multi-Year Research, Development and Demonstration Plan:

- J. Rate of Hydrogen Production

Technical Targets

This project is overcoming the four proton-related major physiological problems that constitute technical barrier J in photobiological H₂ production by genetic insertion of a proton channel into an algal thylakoid membrane. This project is key to achieving the following DOE 2010 and 2015 photolytic biological hydrogen production technical targets:

Characteristics	Units	Current status	2010 target	2015 target
Efficiency of Incident Light Energy to Hydrogen from Water	%	0.1	2	5

Approach

- Overcome technical barrier J by creating a designer alga through genetic insertion of a proton channel into an algal thylakoid membrane for significant (tenfold) improvement in photobiological hydrogen production from water.

Accomplishments

- Accomplished computer-assisted design of DNA sequences for the first set of the envisioned proton-channel genes.
- Synthesized the designed proton-channel genes linked with hydrogenase promoter and thylakoid-signal-polypeptide DNA.

Future Directions

If the required three full-time employee project effort can be fully supported, we anticipate being able to achieve the following milestones in FY 2006:

- Complete the assembly of the constructed hydrogenase promoter-thylakoid signal polypeptide-proton channel gene into a shuttle vector with a selectable marker for *Chlamydomonas reinhardtii* and *E. coli*.
- Accomplish propagation and verification of the DNA sequence for the synthetic hydrogenase promoter-thylakoid signal polypeptide-proton channel gene.
- Achieve genetic transfer of the first hydrogenase promoter-linked polypeptide proton-channel gene (DNA) into a host *Chlamydomonas reinhardtii* strain.

Introduction

Algal (e.g., *Chlamydomonas reinhardtii*) photosynthetic hydrogen (H₂) production from water has tremendous potential as a clean and renewable energy resource. However, a number of technical issues must be addressed before algal H₂ production can become practical. This R&D project is to overcome a major technical barrier, “Rate of Hydrogen Production,” identified in the DOE *Hydrogen, Fuel Cells and Infrastructure Technologies Program Multi-Year Research, Development and Demonstration Plan* for “Molecular Genetics of Organisms for Photobiological Hydrogen Production.” This technical barrier consists of four physiological obstacles that seriously limit the absorbed light energy to hydrogen efficiency. The four physiological obstacles that currently challenge researchers and investors in the field of photosynthetic H₂ production are (1) restriction of photosynthetic H₂ production by accumulation of a proton gradient, (2) competitive inhibition of photosynthetic H₂ production by CO₂, (3) requirement for bicarbonate binding at photosystem II (PSII) for efficient photosynthetic activity, and (4) competitive drainage of electrons by O₂ in algal H₂ production. In order for the photobiological H₂ production system to work, all of these four problems must be solved.

In this project, we will overcome these four technical problems by creating an efficient, robust algal H₂ production system through a novel approach recently developed at Oak Ridge National Laboratory (ORNL). In this approach, a “designer alga” will be created by genetic insertion of hydrogenase promoter-programmed polypeptide proton channels into algal thylakoid membranes. This approach will allow us to simultaneously solve the four physiological problems because all four are related to proton gradient. The success of this work will have a significant impact (a tenfold improvement) on technology development in the field of renewable hydrogen research.

Approach

We have recently developed a systematic approach to create a “super” photosynthetic organism—a designer alga specifically designed for the production of molecular hydrogen through photosynthetic water splitting (ORNL Invention Disclosure ID 0981) [1]. This designer alga will be able to overcome the four proton gradient-related physiological problems that currently challenge researchers and investors in the field of photosynthetic H₂ production. The key element of our proposed approach is creation of a designer alga for efficient, robust production of H₂ through genetic insertion of a programmable polypeptide proton channel into the thylakoid membrane. We propose to

accomplish the genetic insertion of programmable thylakoid-membrane proton channels by transformation of a host alga with a genetic vector that contains a polypeptide proton-channel gene linked with a hydrogenase promoter. The envisioned super alga that can be created by the proposed work should be able to perform autotrophic photosynthesis using ambient-air CO₂ as the carbon source and grow normally under aerobic conditions, such as in an open pond. When the algal culture is grown and ready for H₂ production, the proton-channel gene will then be expressed simultaneously with the induction of the hydrogenase enzyme under anaerobic conditions because of the use of the hydrogenase promoter. The expression of the proton-channel gene should produce polypeptide proton channels in the thylakoid membrane, thus dissipating the proton gradient across the thylakoid membrane without adenosine triphosphate (ATP) formation to enhance H₂ production.

As illustrated in Figure 1, our proof-of-principle experimental studies with the proton uncoupler carbonyl cyanide m-chlorophenylhydrazone (FCCP) have already demonstrated that insertion of a proton-conductive channel in the thylakoid membrane could significantly enhance H₂ production by eliminating the problems of both the proton-gradient accumulation and the newly discovered alternative O₂ sensitivity that is dependent on the proton gradient [2]. Furthermore, the cessation of photophosphorylation (ATP formation) caused by action of the proton channels can, in turn, switch off the Calvin cycle activity (CO₂ fixation), which requires ATP and competes with the ferredoxin (Fd)/hydrogenase H₂ production pathway for the photosynthetically generated electrons. As a result, the competitive inhibition of H₂ production by CO₂ will be eliminated, and photosynthetic H₂ production in the designer alga will be able to occur in the presence of CO₂. Since photosynthetic H₂ production in a successful designer alga no longer requires a CO₂ (HCO₃⁻)-free environment, the requirement for HCO₃⁻ binding at PSII for efficient photosynthetic activity will no longer be a problem. The requirement can be satisfied by leaving some CO₂ in the medium.

The use of polypeptide proton channels in the thylakoid membrane can provide four advantages for

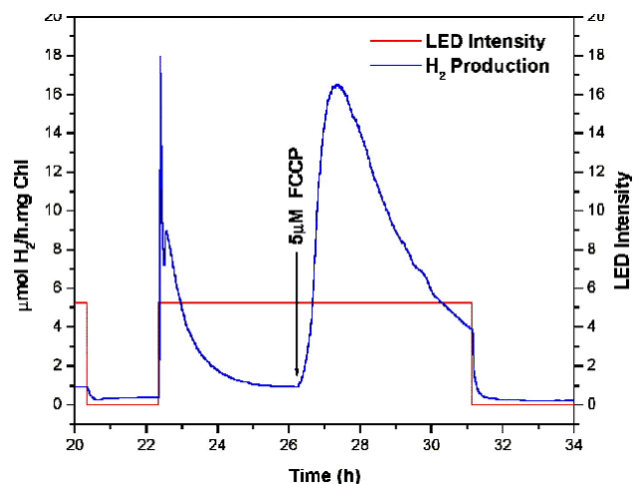


Figure 1. Stimulation of photosynthetic H₂ production in *C. reinhardtii* 137c following addition of the proton uncoupler FCCP in a background atmosphere of 1000 ppm O₂. Addition of 5 μM FCCP produced a dramatic increase in H₂ production, followed by a slow decay. The slow decay is due to a side effect of FCCP, known as “acceleration of the deactivation reactions of the water-splitting system Y”, or ADRY [3], in which FCCP gradually inhibits PSII activity. This ORNL experimental result indicates that use of a polypeptide proton channel that does not have the ADRY effect could enhance H₂ production by eliminating the problems of both the proton-gradient accumulation and the newly discovered alternative O₂ sensitivity. From these data, it can be estimated that application of a polypeptide proton channel could improve the rate of H₂ production by a factor of 10-30.

H₂ photoevolution: (1) the accumulation of a proton gradient that impedes the photosynthetic electron transport from water to Fd/hydrogenase will be prevented; (2) the competitive inhibition of photosynthetic H₂ production by CO₂ (Calvin cycle activity) will be eliminated; (3) the requirement for bicarbonate binding at PSII for efficient photosynthetic activity will be satisfied; and (4) the newly discovered O₂-sensitive pathway (drainage of electrons by O₂) that competes with the H₂-production pathway for photosynthetically generated electrons can be avoided by the dissipation of the proton gradient with the switchable proton channel.

Results

During this reporting period, ORNL made significant progress both in designing the envisioned proton-channel genes and in synthesizing the designed proton-channel genes. Along with our DOE program managers, we helped host the DOE/EERE Biological H₂ Program Working Group meeting at ORNL November 3–5, 2004. Some of our research progress was presented during the meeting.

In technical work in FY 2005, we started with a new and preliminary proton-channel protein design through computer-assisted bioinformatics analysis. Figure 2 presents the molecular structure of this newly designed polypeptide proton channel. In this molecular design, the nanometer pore size for proton conductivity and the molecular stability of the designer proton channel are among the factors that were considered. According to this molecular design, a synthetic gene was then designed by converting the amino acid sequence of the designer

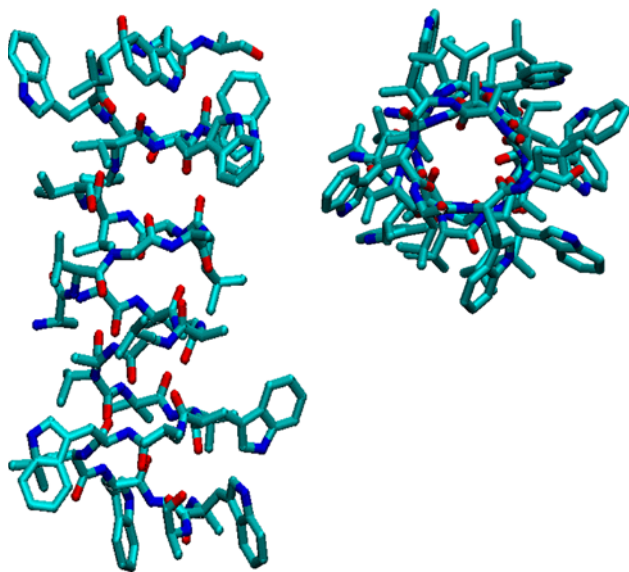


Figure 2. An updated molecular design of a polypeptide proton channel that we accomplished through computer-assisted bioinformatics analysis for this project. The designer proton channel is about 5 nm long (side view shown on the left) with pore size of about 1 nm (top view shown on the right).

protein to a DNA sequence based on the codon usage of the host organism *Chlamydomonas reinhardtii*. Using this approach with bioinformatics tools, we completed the design of the first set of genes (three synthetic genes) that could encode the envisioned switchable proton channels. Figure 3 presents two of the proton-channel genes that we designed. In collaboration with GENEART USA, we recently also completed the physical synthesizing of this set of designer genes. Figure 4 presents a photograph of the DNA materials that we synthesized for the three designer proton-channel genes with GENEART USA. Currently, we are progressing toward verifying the DNA materials for the first set of synthetic genes that can encode the envisioned proton channel for enhanced photobiological H₂ production.

Design No. 1 for Expressing Gramicidin Analog

Hase promoter + *RbcS1* transit peptide + Gramicidin Analog + "natural" 3' UTR
Sequence: 570 bp

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CCCACCGCTGTTTCTCCTGGATTATGGATTTTATACTGGCATCTTTCAAGTC
ACGGAAAAAGCGCGCGCTTCCGACGAAGGTAGGGCTGCACATGGCGAGACC
TGCAGCTCAGCAT-----
CGTTCTCATTCGCGCATTCCTACTGGCGCCTTTAAATGGCAGGACCGCATCCA
AGCTTAAACAATCTGTTCAAATATACAAGTGCcatATGGCCGCCGTCATGCCA
AGTCTCCGCTCTCC-----
GGGTCAAGGCTGCCCGGTGGCTGCCCGGCTCAGGCCAACGAGCCGTGGG
CGCCCTG-----
GTGGCTGTGGCTGTGGGCCcTAAAGCAGTTGACATGTTTGG-----
-----ATGTAACATCCCGTGTGCA---
  
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Design No. 2 for Expressing Melittin

Hase promoter + Plastocyanin transit peptide + Melittin + "natural" 3' UTR
Sequence: 603bp

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CCCACCGCTCTTTCTCCTGGATTATGGATTTTATACTGGCATCTTTCAAGTC
ACGGAAAAAGCGCGCGCTTCCGACGAAGGTAGGGCTGCACATGGCGAGACC
TGCAGCTCAGCAT-----
CGTTCTCATTCGCGCATTCCTACTGGCGCCTTTAAATGGCAGGACCGCATCCA
AGCTTAAACAATCTGTTCAAATATACAAGTGCcatatgaaggtactctgctgccccgcttcc
cgcgcagcgtgtgc-----
gcccgtgcagcgtgaagccgctgctcagcgcgtggcctcggtcgccggtgtgtcggttgcctctctgcccgtgacctg
gctgccacgccGgcacggcgccgtctg-----
-----aagcgcagcagTAAAGCAGTTGACAT-----
-----ATGTAACATCCCGTGTGCA---
  
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Figure 3. Examples of the envisioned proton-channel genes that we designed through computer-assisted bioinformatics analysis. The first proton-channel design (top) is based on the molecular structure of a gramicidin analog, and the second design is based on the structure of melittin.



Figure 4. Photograph of the DNA materials that we synthesized in collaboration with GENEART USA for the three proton-channel genes that we designed. We are in progress toward gene transformation to create the envisioned designer alga for enhanced photobiological H_2 production from water.

Conclusions

Creation of designer alga by genetic insertion of a proton channel into a thylakoid membrane is one of the key R&D tasks required for the photobiological H_2 production system to work. We have already developed a systematic approach to achieving the proposed work. The proof-of-principle FCCP experiments demonstrated that use of the envisioned switchable proton channel could result in significant (10–30 fold) improvement in photobiological H_2

production from water. We have now completed the molecular design and physical synthesizing of the DNA materials for the first set of the designer proton-channel genes. Work is needed to transfer these synthetic proton channel genes into the host organism *Chlamydomonas reinhardtii* to create the envisioned proton-channel designer alga for enhanced photobiological H_2 production from water.

FY 2005 Publications/Presentations

1. James W. Lee, Dong Xu, Barbra Evans, Laurie Mets, Jizhong Zhou, Bin Zhao, and Weimin Gao, "Creation of Designer Alga for Efficient and Robust Production of H_2 ," presented at the DOE Hydrogen, Fuel Cells & Infrastructure Technologies Program Merit Review meeting, May 23–26, 2005, Washington, D.C.
2. James W. Lee, "Genomic Biotechnology: Creation of Designer Alga for Enhanced H_2 Production from Water," presented for the DOE-sponsored Jason Study on biological hydrogen and ethanol production, July 1, 2005, San Diego, California.

References

1. J. W. Lee and E. Greenbaum, "Method for Creating Efficient and Robust Photosynthetic H_2 -production Systems," ORNL Invention Disclosure 0981, 2001.
2. J. W. Lee and E. Greenbaum, "A New Oxygen Sensitivity in Photosynthetic H_2 Production," *Applied Biochemistry and Biotechnology*, **105–108**, 303–313 (2002).
3. V. D. Samuilov, E. L. Barsky, and A. V. Kitashov, "ADRY Agent-induced Cyclic and Non-cyclic Electron Transfer around Photosystem II," pp. 267–270 in *Photosynthesis: From Light to Biosphere*, P. Mathis, ed., Vol. II, Kluwer Academic Publishers, The Netherlands, 1995.